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# Construction and Analysis of Fractional Multifactorial Designs To Study Attachment Strength and Transfer of *Listeria monocytogenes* from Pure or Mixed Biofilms after Contact with a Solid Model Food

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**The aim of this study was to establish which of seven factors influence the adhesion strength and hence bacterial transfer between biofilms containing *Listeria monocytogenes* (pure and two-species biofilms) and tryptone soya agar (TSA) as a solid organic surface. The two-species biofilms were made of *L. monocytogenes* and one of the following species of bacteria: the nonpathogenic organisms *Kocuria varians*, *Pseudomonas fluorescens*, and *Staphylococcus sciuri* and CCL 63, an unidentified gram-negative bacterium isolated from the processing plant environment. We used biofilms prepared under conditions simulating open surfaces in meat-processing sites. The biofilm's adhesion strength and population were evaluated by making 12 contacts on a given whole biofilm (4.5 $cm^2$), using a new slice of a sterilized TSA cylinder for each contact, and plotting the logarithm CFU · $cm^{-2}$ detached by each contact against the contact number. Three types of detachment kinetics were observed: biphasic kinetics, where the first slope may be either positive or negative, and monophasic kinetics. The bacteria that resisted a chlorinated alkaline product and a glutaraldehyde- and quaternary ammonium-based disinfectant had greater adhesion strengths than those determined for untreated biofilms. One of the four non-*Listeria* strains studied, *Kocuria varians* CCL 56, favored both the attachment and detachment of *L. monocytogenes*. The stainless steel had smaller bacterial populations than polymer materials, and non-*Listeria* bacteria adhered to it less strongly. Our results helped to evaluate measures aimed at controlling the immediate risk, linked to the presence of a large number of CFU in a foodstuff, and the delayed risk, linked to the persistence of *L. monocytogenes* and the occurrence of slightly contaminated foods that may become dangerous if *L. monocytogenes* multiplies during storage. Cleaning and disinfection reduce the immediate risk, while reducing the delayed risk should be achieved by lowering the adhesion strength, which the sanitizers used here cannot do at low concentrations.**

Microorganisms in foods may be derived from the raw materials, ingredients, personnel, or the work environment. Epidemics of listeriosis, which is rare but serious when it affects the so-called at-risk population (43), have been traced back to environmental contamination of food processing areas (48). *Listeria monocytogenes* genotypes, absent from the raw materials, may be repeatedly found in the finished products and on work surfaces (3). It is therefore interesting to identify the factors that affect adhesion strength and hence transfer of *L. monocytogenes* from an inert surface to a food following contact. Such data can be useful for quantitative analysis of the risks associated with ready-to-eat foods that may be contaminated during manufacture (11, 18). Most studies of detachment of microbial cells use biofilms formed under dynamic conditions in which detachment is induced by liquid flow. These studies have shown the influence of numerous factors on the detachment of bacterial cells, such as nutrient limitation (21, 38, 45), growth phase (38), growth rate (40, 41), shape of the microbial cells (20), and nature of the substrate material (20, 40, 46). Other authors have studied the transfer of bacterial cells simply deposited on a surface (36, 37). Montville and Schaffner (36) used a procedure where the substrates were contaminated and the transfer during food handling was simulated. They showed that the transfer coefficient (TC) (CFU on the target surface/CFU of the source surface) was lower for higher levels of inocula.

We have studied the transfer, through contact with a solid food model, of cells from biofilms formed under static conditions representative of biofilm formation in the food industry. We have previously demonstrated the influence of three factors: substrate material, bacterial species, and prior contact with a sanitizer (34, 35). Previously, we demonstrated that in pure culture *L. monocytogenes* adhered more strongly to polychloride vinyl or polyurethane conveyor belt materials than three other non-*Listeria* bacterial strains of food industry origin (35). However, it is not known how *L. monocytogenes* behaves when combined with other bacteria, a situation that simulates industrial conditions better than pure cultures. Our aim in the present study was to determine the factors impacting the transfer of *L. monocytogenes* from pure or bimicrobial biofilm (the nature of the non-*Listeria* strain associated with *L. monocytogenes* being one the factors studied) and the transfer of the associated non-*Listeria* strains. Four new factors were studied simultaneously with the three others investigated in

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TABLE 1. Levels of the factors used in the fractional factorial design for biofilms of *L. monocytogenes* combined with one of the four strains indicated in the table

| Factor[a] | Level | | | |
|---|---|---|---|---|
| Material (m) | Stainless steel | Polyurethane PU1 | Polyurethane PU2 | Polyvinyl chloride (PVC) |
| Chemical shock (ch) | Galorox JH | Galox Azur | None | None |
| Bacterial strain (s) | *Staphylococcus sciuri* CCL 101 | *Pseudomonas fluorescens* CCL 134 | *Kocuria varians* CCL 56 | Strain CCL 63 |
| Glucose (mmol liter$^{-1}$) (glu) | 0 | 50 | | |
| Biofilm age (day) (age) | 1 | 2 | | |
| $CaCl_2$ (mmol liter$^{-1}$) (calc) | 0 | 7.5 | | |
| Temperature (temp) | 15°C | 25°C | | |

[a] Abbreviations of the names of the factors are given in parentheses.

our previous work: (i) addition of glucose to biofilm culture medium, (ii) addition of calcium to biofilm culture medium, (iii) biofilm incubation temperature identical to that of the food model used for the contacts, and (iv) age of the biofilms.

(This work forms part of G. Midelet's Ph.D. thesis research at the University of Bourgogne, Dijon, France.)

## MATERIALS AND METHODS

**Test surfaces.** Four materials were used (as 15-mm by 30-mm slides): stainless steel (2 RB finish, AISI 304; British Steel, Inox Industrie, Aulnay-sous-Bois, France), polyvinyl chloride (PVC) (NONEX 2 M 1320; Ammeraal, Seclin, France), and two polyurethanes (PU) with different water contact angles: 83° ± 3° for ROPANYL 2 M 1795 (or PU1) and 75° ± 5° for 2 M 1719 (or PU2) (Ammeraal). The slides were washed with 2% (vol/vol) $RBS_{35}$ (Traitement chimique des surfaces; Frelinghien, France), an alkaline solution, and rinsed repeatedly as previously described (27). The stainless steel was autoclaved, and the PU and PVC were sterilized by immersion in 300 ml of 0.2% (vol/vol) peracetic acid solution (OXYGAL NEP; Penngar, Vaas, France) for 5 min at room temperature. The slides were rinsed in 300 ml sterilized ultrapure water and dried in a laminar airflow hood.

**Bacterial strains.** Thirteen strains of bacteria, previously isolated from food processing surfaces (8), were screened for biofilm surface coverage when grown under conditions that mimic open surfaces in the meat industry (33). We selected four non-*Listeria* strains (two gram-positive and two gram-negative strains) whose biofilms differed in surface coverage and also in influence on the adherent population of *L. monocytogenes* (8, 27, 33). *Staphylococcus sciuri* CCL 101 was isolated from the floor of a catering establishment. *Pseudomonas fluorescens* CCL 134 was isolated from cheese-making premises; we obtained it from the Société de Recherche et de Développement Alimentaire Bongrain (La Boissière-Ecole, France), where it was referenced as D32.2. *Kocuria varians* CCL 56 was isolated from a gasket on a milk pasteurization line. Strain CCL 63, an unidentified gram-negative, oxidase-positive, catalase-positive rod, was isolated from a conveyor belt in a cured meat product establishment. *Listeria monocytogenes* A, also referred to as CCL 128, was isolated from a dairy environment; we obtained it from the Société de Recherche et de Développement Alimentaire Bongrain. It was chosen because its restriction fragment length polymorphism profile was the most representative of *L. monocytogenes* strains found in dairy premises (C. Martiré, personal communication). This *L. monocytogenes* strain, a 4b serotype, has already been used in several previous studies (5, 8, 27, 29, 35). Cultures were maintained for no more than 1 month on tryptone soya agar slopes (TSA; Difco, Le Pont de Claix, France) at 3°C. The slopes were inoculated from long-shelf-life stock cultures grown in a medium containing meat extract (Difco), 3 g liter$^{-1}$; Bacto tryptone (Difco), 5 g liter$^{-1}$; and glycerol (Fisher Scientific Labosi, Elancourt, France), 150 g liter$^{-1}$, and stored in liquid nitrogen.

**Construction of fractional factorial designs.** To study the influence of the seven factors cited above on bacterial transfer, we used two asymmetrical fractional factorial designs with a resolution of 4. The first one included biofilms with *L. monocytogenes* alone, the second included biofilms of *L. monocytogenes* associated with a non-*Listeria* strain. Table 1 shows the factors and their levels for this second design, which has 64 units. The four last factors have two levels, while the three first ones have four levels. This introduced a dissymmetry between factors, and this is why the design is said to be asymmetrical. Moreover, as no more than 16 experiments can be made simultaneously, the 64 units are divided into four blocks of 16 units each. The biofilms of each block were all prepared at the same temperature.

For the construction of the 64-unit design, mixed biofilms were studied and factors with four levels were decomposed into two-level pseudofactors, as exemplified in Table 2, which shows material decomposed into m1 and m2 (1). That decomposition leads to the pseudofactors m1 (where "m" is a designation for material), m2, ch1 (where "ch" stands for chemical shock), ch2, s1 (where "s" stands for bacterial strain), s2, and bl1 (where "bl" indicates blocks), and bl2. The levels of the other two-level factors are coded −1 and 1. Then, the design is built using defining relations as follows. Temperature is defined as $m2 \cdot s2 \cdot ch2$, glucose is $m2 \cdot s1 \cdot s2 \cdot ch1$, age is $m1 \cdot s1 \cdot s2 \cdot ch2$, calcium is $m1 \cdot m2 \cdot s1 \cdot ch1 \cdot ch2$, bl1 is $m1 \cdot m2 \cdot s2 \cdot ch1$, and bl2 is $m1 \cdot ch1 \cdot ch2$. For instance, if m1 = 1, m2 = 1, s1 = 1, s2 = −1, ch1 = 1, and ch2 = −1, the level of temperature is 1 (1 × −1 × −1) and that of glucose is −1 (1 × 1 × −1 × 1), etc.

These defining relations were found by the software PLANOR (A. Kobilinsky, INRA, Jouy en Josas, France) (25) to get a resolution 4 design, which is a design which allows estimation of all main effects in the model, including all two-factor interactions. It was also required that the main effects, different from the temperature, be unaliased (or unconfounded) with the blocks. The backtrack search algorithm used by PLANOR originates from the methodology initially proposed (17) to find suitable design keys (39). The directive, FKEY of GENSTAT (39a), uses exactly the same search algorithm and the program, FACTEX of SAS QC (44), a very similar one. These last two programs could have been used to find a resolution 4 fraction. However, PLANOR can make the search in several completely different random orders, thus leading to an assortment of resolution 4 solutions, from which one can select the more appropriate design. Here, the above solution was selected because it allows, besides the estimation of main effects, the estimation of 29 degrees of freedom (df) of the two-factor interactions, whereas the other solutions can estimate only between 19 and 24 df of these interactions. Table 3 lists these combinations, as well as the 29 interactions that are not aliased. It is to be noted that the nature of aliasing for interactions depends on the parameterization of the model. As explained by Kobilinsky (24), it is very plain if the decomposition into degrees of freedom is the one naturally associated with the pseudofactors that were used for the construction. This decomposition was used here to explicit the alias. As the factor "chemical shock" has only three levels instead of four, one of the level "none" is hence used twice on 32 units. This kind of modification (first considered by Addelman) (1) induces a slight modification in the aliasing. There is one more residual degree of freedom and three more estimable interactions, and the number of terms in the sum of aliased interactions is reduced for some of them. Again, the nature of alias remains quite plain if, to characterize the "chemical shock" effect, the decomposition into degrees of freedom introduces one contrast between the two

TABLE 2. Decomposition of factor "material" into two pseudofactors, m1 and m2

| Material | Pseudofactor | |
|---|---|---|
| | m1 | m2 |
| Steel | 1 | 1 |
| PU1 | 1 | −1 |
| PU2 | −1 | 1 |
| PVC | −1 | −1 |

TABLE 3. Aliasing in the design with 64 units for mixed cultures[a]

| |
|---|
| Nonaliased interactions |
| m2 · s1, m1 · s2, m1 · s1, m1 · m2 · s2, m1 · m2 · s1 · s2, m2 · ch1 · ch2, m1 · ch1, m1 · m2 · ch2, m1 · m2 · ch1, m1 · temp, m2 · age, m2 · calc, m1 · calc, m1 · glu, s2 · ch1, s2 · ch1 · ch2,s1 · ch2, s1 · ch1, s1 · s2 · ch1 · ch2, s1 · s2 · temp, s2 · age, s2 · calc, s1 · s2 · calc, s2 · glu, ch1 · temp, ch1 · age, ch2 · calc, ch1 · calc, ch2 · glu |
| Estimable linear combinations including a block effect |
| bl1, temp − bl1 · bl2, −m1 · ch1 · ch2 + bl2 |
| Estimable linear combinations of several interactions |
| m2 · s2 + ch2 · temp, m2 · s1 · s2 − ch1 · glu, m1 · s1 · s2 − ch2 · age, m1 · m2 · s1 + ch1 · ch2 · calc − temp · age, m2 · ch2 + s2 · temp, m2 · ch1 − s1 · s2 · glu, m1 · ch2 − s1 · s2 · age, m1 · m2 · ch1 · ch2 + s1 · calc + age · glu, m2 · temp + s2 · ch2, m1 · m2 · temp − s1 · age − calc · glu, m1 · age − s1 · s2 · ch2, m1 · m2 · age − s1 · temp + ch1 · ch2 · glu, m1 · m2 · calc + s1 · ch1 · ch2 − temp · glu, m2 · glu − s1 · s2 · ch1, m1 · m2 · glu + ch1 · ch2 · age − temp · calc, s1 · glu − ch1 · ch2 · temp + age · calc |

[a] See Table 1 for explanation of abbreviations.

active shocks and another between the absence of shock (level "none") and the mean effect of the two active shocks.

The design with 32 units and *L. monocytogenes* alone is built in the same manner as the one with 64 units and mixed culture. But, in that case, only one resolution 4 regular fraction exists, up to permutations between the four two-level factors on one side and between the two four-level factors on the other side. The found solution is defined as follows: age = m2 · ch1 · glc, temperature = m1· ch2 · glc, and cal = m1 · m2 · ch1 · ch2 · glc.

The only block factor coincides with the temperature factor. It means that since the temperature remains constant on each block, it is impossible to distinguish between its effect and the block effect. Again, two levels of the couple (ch1 and ch2) are associated with the same level "none" [(1, 1), (1, −1)], while the two others are associated with Galorox JH 3 and Galox Azur. Using two other levels, for instance, (−1, 1) and (1, −1) for "none" instead of (1, 1) and (1, −1) would give the same nature of aliasing between factorial effects. Thus, the interaction between the material and the chemical shock has five unaliased degrees of freedom. The other two-factor interaction effects are aliased by pair, excepted three of them (the corresponding estimable function is mat^2 · choc + glc · age + cal · temperature).

**Biofilm development.** The refrigerated cultures were transferred to TSA slopes and incubated for 24 h at 25°C for the four non-*Listeria* species and at 37°C for *L. monocytogenes.* The bacteria were then washed twice by centrifugation at 2,800 × *g* for 10 min in 9 ml physiological saline. The concentration of the suspension was adjusted to $10^8$ CFU $ml^{-1}$ (optical density at 600 nm = 0.15 in 1.5-cm-diameter tubes), for non-*Listeria* bacteria and $10^6$ CFU $ml^{-1}$ for *L. monocytogenes*. Sterilized slides were adhered to the bottoms of 50-cm-diameter petri dishes with double-sided adhesive tape (Tesa; Foto Film, France). To prevent dehydration of the biofilms during incubation, each 50-cm petri dish was placed in a 120-cm petri dish containing 25 ml of water. Meat exudate was obtained by thawing a deep-frozen shoulder of beef, as previously described (35). Seven milliliters of meat exudate was deposited on each slide with a pipette. Half of the slides were left at 25°C and the other half were left at 15°C for 75 min. The meat exudate was then removed by pipetting, and 7 ml of either the non-*Listeria* bacterial suspension ($10^8$ CFU ml per liter) or of physiological saline (for the pure culture *L. monocytogenes* biofilms) was deposited on each slide. The slides were then kept for 3 h at 25°C or 15°C to allow adhesion of the non-*Listeria* cells. The nonadhering bacteria or the physiological saline (from slides used to grow pure *L. monocytogenes* biofilms) was removed by pouring 25 ml peptone solution (1 g $liter^{-1}$ of Bacto peptone; Difco). Seven milliliters of *L. monocytogenes* suspension ($10^6$ CFU $ml^{-1}$) was deposited on each slide. The slides were then kept for 3 h at 25°C or 15°C to allow adhesion of *L. monocytogenes*. The nonadhering bacteria were removed by pouring of 25 ml peptone solution before incubation at 25°C or 15°C for 17 h (1-day biofilms) or 41 h (2-day biofilms).

**Biofilm structure enhancement.** In half of the biofilms, in accordance with the defined experimental design constructed, $CaCl_2$ dihydrate (Merck Eurolab, Strasbourg, France) and/or glucose was added to the physiological saline, the bacterial suspension, the meat exudate, and the peptone solution to a final concentration of 7.5 mmol $liter^{-1}$ for $CaCl_2$ and 50 mmol $liter^{-1}$ for glucose. The 50-mmol $liter^{-1}$ $CaCl_2$ concentration was chosen because it increased surface coverage of the four non-*Listeria* strains studied and stimulated microcolony formation in *S. sciuri* CCL 101 and *P. fluorescens* CCL 134 biofilms (20). The glucose concentration of 50 mmol $liter^{-1}$ was chosen, as scanning electron microscopy indicated that it promotes the formation of microcolonies in biofilms of *P. fluorescens* CCL 134 and the production of exopolymers by strain CCL 63 (33).

**Chemical shocks.** A certified cleaning and disinfection agent (Galorox JH; Penngar) containing sodium hydroxide and sodium hypochlorite and a certified disinfectant (Galox Azur; Penngar) made of glutaraldehyde and quaternary ammonium compounds were used at concentrations of 0.25 and 0.05% (wt/vol), respectively, to cause approximately one decimal reduction of the biofilm population after 5-min contact time (33). These concentrations are far below these recommended of between 2 and 5% for Galorox JH and between 1 and 3% for Galox Azur. It is of interest to note that product efficacy measurements based on residual CFU counts do not distinguish between detachment and killing of bacterial cells. Six milliliters of the solutions was deposited on a 1-day or 2-day biofilm and left for 60 s. After the Galorox JH or the Galox Azur solution was removed by pipetting, 7 ml of one of the following neutralizing solutions was deposited: 17-g $liter^{-1}$ sodium thiosulfate (Sigma, Saint-Quentin Fallavier, France) on the biofilm treated by Galorox JH or 30-g $liter^{-1}$ soy lecithin solution (Sigma) and 3-g $liter^{-1}$ L-histidine (Fisher Scientific Labosi) on the biofilm treated by Galox Azur. These solutions had previously been validated to confirm that they neutralize antimicrobial activity under the conditions used here. After a 5-min contact, the biofilm was rinsed with 25 ml peptone solution and adjusted or not to 7.5 mmol · $liter^{-1}$ $CaCl_2$ and/or to 50 mmol · $liter^{-1}$ glucose.

**Quantification of CFU transferred from biofilm to a solid model food.** TSA has been shown to mimic meat effectively in terms of bacterial cells transferred after contact with a biofilm (33). Syringes with a 40-mm inner diameter, as previously described (34), were filled with melted TSA, which was left to solidify overnight at 15°C or 25°C so that the contact between TSA and biofilms occurred at the same temperature as the biofilm culture incubation. The column of solidified TSA was pushed to the end of the cylinder with the plunger. A syringe, topped with a weight so that the whole (syringe plus weight) weighed 500 g, was then placed on a biofilm and was left for 30 s. This 500-g mass was the same as used in a previous study in which pieces of meat were placed on biofilms (35). About 3 mm of solidified TSA was then pushed out from the end of the syringe cylinder, and a portion was cut off with a sterilized knife. Contacts between TSA and biofilm were made 12 times in succession on the same biofilm. TSA completely covered the slides and so was always in contact with the whole biofilm. The numbers of bacterial CFU transferred by the first eight contacts and the last two contacts were determined as follows (CFU transferred at contacts 9 and 10 were not counted because they do not contribute too much in the slope calculation). Each piece of TSA was put in a bottle containing 10 ml of peptone solution. The bottle was vortexed for 20 s to detach bacterial cells. CFU counts were made using a spiral plater (Spiral System DS; Interscience, Saint Nom la Bretèche, France) on the appropriate medium after appropriate decimal dilution with peptone solution.

**Medium for enumeration of microorganisms.** *L. monocytogenes* was enumerated using Palcam agar (AES, Combourg, France) (48 h at 37°C). Non-*Listeria* microorganisms were enumerated on TSA containing an antibiotic (Sigma): 1-μg $ml^{-1}$ novobiocin for *S. sciuri*, 50-μg $ml^{-1}$ ampicillin for *P. fluorescens*, 50-μg $ml^{-1}$ furazolidone for *K. varians*, and 1-μg $ml^{-1}$ ampicillin for strain CCL 63. Incubation was at 30°C for 24 h.

**Assessment of attachment strength and biofilm population.** Two-phase curves were obtained by plotting the logarithm of the number of CFU transferred by each contact against the contact number. Previous work (35) showed that by fixing the breakpoint of the two-phase curves at transfer number 3, we obtained slopes $k_1$ (the slope of the first part of the biphasic transfer curve) and $k_2$ (the slope of the second part of the biphasic transfer curve), which were similar to those estimated by nonlinear regression with the Superfit program without fixing the breakpoint (9). Except for 1 of 160 cases, where the fourth contact was used as a breakpoint, the slopes calculated by fixing the breakpoint at transfer number 3 were successfully used to assess attachment strength and biofilm population using the Veulemans et al. (49) equation as previously described (35):

$$\sum_{n=1}^{n\to\infty} N_n = N_1 + N_2 + \frac{N_3}{(1 - 10^{k_2})}$$

where $n$ is the contact number and $N$ is the number of CFU detached at contact number $n$.

A transfer coefficient, which is also an indicator of adhesion strength, was calculated by dividing the total number of CFU detached during the first eight contacts by the biofilm population calculated using the above formula.

**Statistical analysis.** The factorial designs were analyzed using ANALYS, version 2.2 (23). The model used to perform a first analysis of variance took into

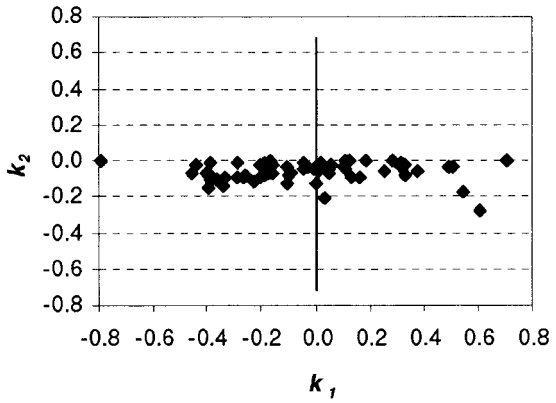


FIG. 1. Slope $k_2$ as a function of slope $k_1$ in curves of the logarithm of CFU $cm^{-2}$ of *L. monocytogenes* detached by contact of a mixed biofilm with a solid food model versus the contact number.

account the main effects of all the factors and the interactions of any two factors. ANALYS takes into account the aliasing between some interactions and provides the linear combinations of those that can be estimated and a significance for each main effect, each estimable interaction, and these linear estimable combinations of interactions. To obtain this efficiency value, the *t* or *F* test used a residual variance based here on only 4 df, but a complementary Bayesian "Box and Meyer" analysis was also performed to complement the analysis of variance. In many cases, the terms or linear combinations that do not appear significant at all (>25%) were removed from the model. This 25% threshold is arbitrary, but it is sufficiently high to limit the risk of an active effect being taken off, which would not be the case if the classical (also arbitrary) 5% threshold had been chosen. This increased the number of residual degrees of freedom and slightly modified the tests without changing the interpretation.

## RESULTS

**Adhesion strength.** Figure 1 gives the values of $k_2$ as a function of $k_1$ for each of the 64 curves obtained in experiments with *L. monocytogenes* in mixed cultures. This figure (like the curves [not shown] for *L. monocytogenes* in pure culture and non-*Listeria* strains associated with *L. monocytogenes*) demonstrates (i) that there was no relation between $k_1$ and $k_2$, (ii) that the slope $k_1$ covered a much greater range of values than the slope $k_2$, (iii) that the $k_2$ slope was often close to zero, and (iv) that the slope $k_1$ could be positive. We could therefore distinguish three types of curves: (i) curves where $k_1$ was positive, as in Fig. 2a; (ii) those where $k_1$ was negative and $k_1$ was lower than $k_2$, as in Fig. 2b; and (iii) those where $k_1 = k_2$, as in Fig. 2c. The difference between $k_1$ and $k_2$ was <0.05 in 12%, 14%, and 38% of the experimental units for *L. monocytogenes* in pure biofilms, *L. monocytogenes* in mixed biofilms, and non-*Listeria* strains associated with *L. monocytogenes*, respectively. The most frequent case was the second one (Fig. 1). When the slope $k_1$ was negative, it was rare for $k_1$ to exceed $k_2$, but when it did, the difference between the two slopes was very small and the curve could be considered monophasic. The population corresponding to the first three points of the curves (characterized by the slope $k_1$) was generally smaller than the population corresponding to the second part of the curves and represented a mean of 29, 33, and 40% of the population calculated by the Veulemans et al. equation (49) for *L. monocytogenes* in pure biofilms, *L. monocytogenes* in mixed biofilms, and non-*Listeria* strains associated with *L. monocytogenes*, respectively.

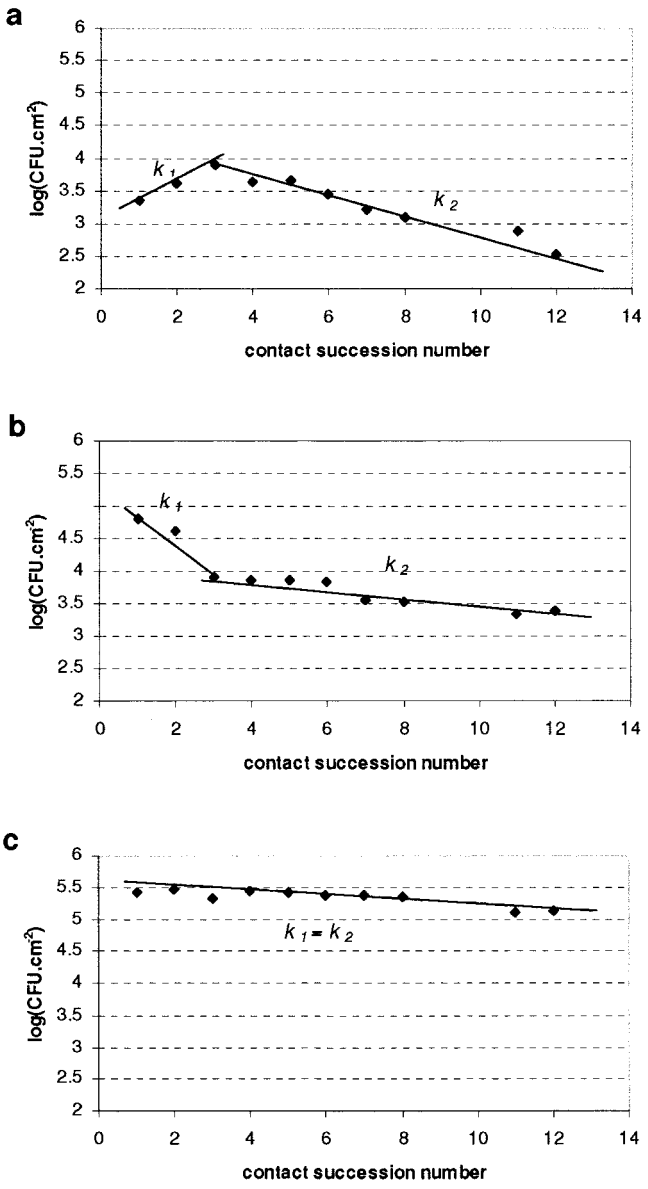


FIG. 2. Example of each of the three types of detachment kinetics observed. These curves plot the logarithm of the number of CFU $cm^{-2}$, detached from a biofilm through contact with a solid food model, against the contact order. (a) Biphasic curve, where the first slope $k_1$ is positive. This is a 1-day biofilm of *K. varians* grown at 25°C with added glucose on stainless steel and then subjected to a chemical shock with Galorox JH 3 (a chlorinated alkaline product). (b) Biphasic curve where the value for $k_1$ is negative and below the value for $k_2$. The results are from a 1-day biofilm of *P. fluorescens* grown at 15°C with added glucose and calcium on polyurethane (PU2). (c) Monophasic curve ($k_1 = k_2$). These results are from a 2-day biofilm of *K. varians* grown at 25°C with added glucose and calcium on polyurethane (PU1).

Finally, Fig. 3a shows that it was mainly the slope $k_2$ that determined the number of contacts needed for just 1 CFU · $cm^{-2}$ to remain on the surface. The biofilm population (Fig. 3b) and the slope $k_1$, characterizing the first three contacts, had a negligible role. In Fig. 3a, we can see, for instance, that with a $-0.05$ $k_2$ slope, 82 contacts were necessary to have only 1 CFU · $cm^{-2}$ remaining on the surface.

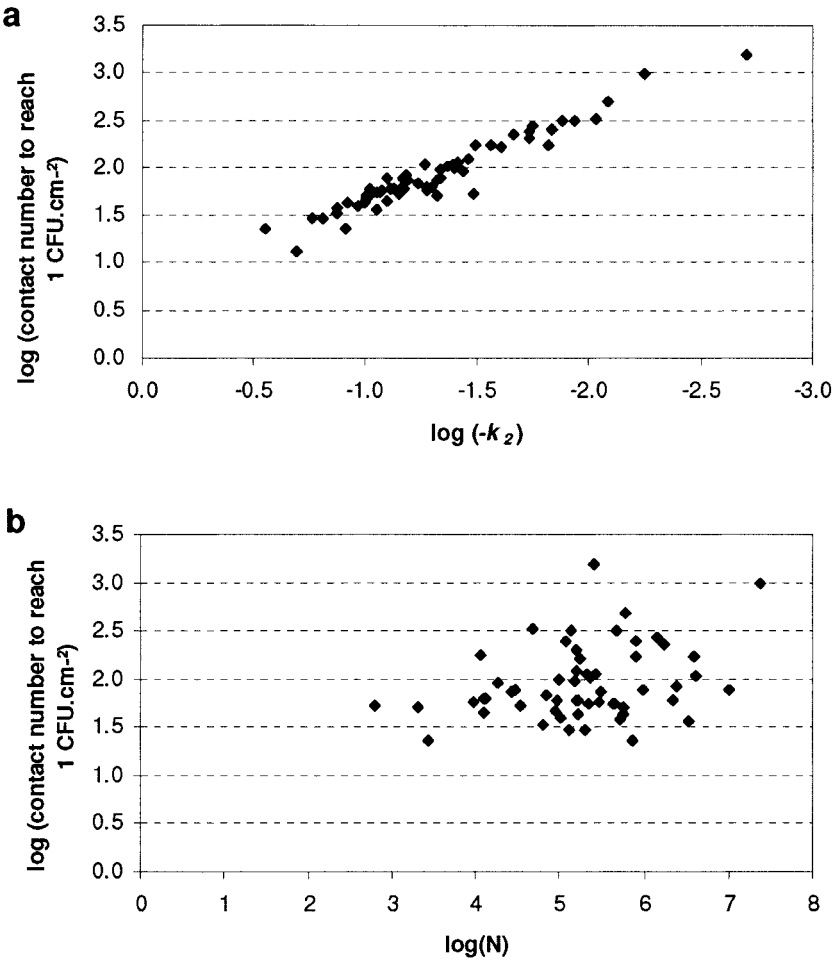


FIG. 3. Logarithm of the number of contacts needed to reach 1 CFU $cm^{-2}$ of *L. monocytogenes* on the biofilm support as a function of the log $(-k_2)$ (a) and the logarithm of the *L. monocytogenes* population in mixed biofilms (b).

Four factors had a significant effect on at least one of the nine responses studied to characterize the adhesion strength ($k_1$, $k_2$, and the transfer coefficient calculated for the detachment of *L. monocytogenes* in pure and mixed culture and for the detachment of the non-*Listeria* strains associated with *L. monocytogenes*). Table 4 shows only main effects. The interactions were omitted for two reasons. First, they or their linear combinations appeared not to be significant or to be much less important than the main effects. Then, because of the aliasing, it was difficult to get the associated means without making further assumptions about which of the aliased interactions were negligible. The only main effects kept were those which were clearly active for several of the analyzed responses. Four factors were of no significance and therefore were not included in Table 4: (i) the temperature of biofilm growth (two temperatures used in industrial work areas were compared), (ii) the addition of calcium, (iii) the addition of glucose, and (iv) biofilm age. We will describe below the main effects of each of the three impacting factors.

**Non-*Listeria* strains.** *L. monocytogenes* had a mean transfer coefficient of 0.78 when associated with *K. varians* and 0.55 in pure culture, a significant difference (mean comparison, $P < 0.05$). The values of $k_2$ also showed that the adhesion strength of *L. monocytogenes* was low in the presence of *K. varians.*

**Substrate material.** The slope $k_2$ of the biphasic curves obtained with the non-*Listeria* strains, associated with *L. monocytogenes*, and their transfer coefficient showed that adhesion strength was lower on stainless steel than on polymers.

**Chemical shock.** Chemical shocks led to positive slope $k_1$ for *L. monocytogenes*, whether in pure or mixed culture, and for the non-*Listeria* strains but only after the action of chlorinated alkaline product. The transfer coefficient and/or the slope $k_2$ showed that adhesion strength was greater after chemical shock. The differences in these two parameters between untreated and treated biofilms were significant, except for pure biofilms of *L. monocytogenes* treated with glutaraldehyde-based product.

Calcium and the temperature of biofilm growth and of the substrate during contact appeared to have no significant influence on any of the responses studied. This indicates that the block factor, aliased with the temperature factor, incorporated when making the two-block design, had no significant effect; so there was no influence on the results, even if the experiments were not done on the same day.

TABLE 4. Means for the different indicators of adhesion strength, $k_1$, $k_2$, and TC of *L. monocytogenes* in pure or mixed culture or strains associated with *L. monocytogenes*, for each of the levels of the factors with a significant effect on these parameters[a]

| Factor | Mixed biofilm | | | | | | Pure *L. monocytogenes* biofilms | | |
|---|---|---|---|---|---|---|---|---|---|
| | *L. monocytogenes* | | | Other strains | | | | | |
| | $k_1$ | $k_2$ | TC | $k_1$ | $k_2$ | TC | $k_1$ | $k_2$ | TC |
| General mean | −0.038 | −0.062 | 0.62 | −0.080 | −0.074 | 0.65 | 0.009 | −0.057 | 0.55 |
| Material | | | | | ** | * | | | |
| Stainless steel | | | | | −0.097 a | 0.75 b | | | |
| PU1 | | | | | −0.071 ab | 0.64 ab | | | |
| PU2 | | | | | −0.058 b | 0.52 a | | | |
| PVC | | | | | −0.069 b | 0.69 ab | | | |
| Chemical shock | ** | | | * | *** | ** | ** | ** | * |
| None | −0.194 a | | 0.72 b* | −0.150 a | −0.095 a | 0.78 c | −0.136 a | −0.074 a | 0.68 b |
| Galox Azur | 0.123 b | | 0.51 a | −0.077 ab | −0.064 b | 0.58 b | 0.163 b | −0.060 ab | 0.49 ab |
| Galorox JH | 0.114 b | | 0.52 a | 0.055 b | −0.040 b | 0.48 a | 0.146 b | −0.022 b | 0.33 a |
| Strain | | * | * | | * | | | | |
| *K. varians* | | −0.090 a* | 0.78 b | | −0.066 b | | | | |
| CCL 63 | | −0.062 ab | 0.65 ab | | −0.109 a | | | | |
| *S. sciuri* | | −0.057 ab | 0.57 ab | | −0.051 b | | | | |
| *P. fluorescens* | | −0.037 b | 0.48 a | | −0.069 b | | | | |

[a] Results were obtained in the fractional factorial design. ***, effect significant at the 1% level; **, effect significant at the 1% level; *, effect significant at the 5% level. When the same letter is associated with two levels of a factor, this indicates that the means for each of these two levels are not significantly different at the 5% level. Residual standard deviations ranged from 0.173 (11 df) to 0.220 (14 df) for $k_1$, 0.027 (10 df) to 0.041 (8 df) for $k_2$, and 0.158 (3 df) to 0.235 (8 df) for TC.

TABLE 5. Mean biofilm populations (log CFU $cm^{-2}$) estimated by the Veulemans equation for each of the levels of the factors with a significant effect on the *L. monocytogenes* population in mixed or pure culture and on the population of strains associated with *L. monocytogenes*[a]

| Factor | Mixed biofilms | | Pure *L. monocytogenes* biofilms |
|---|---|---|---|
| | *L. monocytogenes* | Other strains | |
| General mean | 5.3 | 6.3 | 5.7 |
| Material | *** | ** | ** |
| Stainless steel | 4.6 a | 5.7 a | 4.5 a |
| PU1 | 5.7 b | 6.5 b | 6.2 b |
| PU2 | 5.3 b | 6.7 b | 5.5 b |
| PVC | 5.4 b | 6.4 ab | 5.9 b |
| Chemical shock | ** | *** | ** |
| None | 5.6 b | 6.8 b | 6.2 b |
| Galox Azur | 5.0 a | 6.1 a | 5.1 a |
| Galorox JH | 4.7 a | 5.6 a | 5.3 a |
| Strain | ** | *** | |
| *K. varians* | 5.7 c | 5.8 a | |
| CCL 63 | 4.7 a | 7.0 b | |
| *S. sciuri* | 5.1 ab | 5.9 a | |
| *P. fluorescens* | 5.5 bc | 6.7 b | |
| Glucose (mmol $liter^{-1}$) | * | | |
| 0 | 5.1 a | | |
| 50 | 5.4 b | | |
| Biofilm age (days) | | * | * |
| 1 | | 6.1 a | 5.1 a |
| 2 | | 6.5 b | 6.0 b |

[a] Results were obtained in the fractional factorial design. ***, effect significant at the 1% level; **, effect significant at the 1% level; *, effect significant at the 5% level. When the same letter is associated with two levels of a factor, this indicates that the means for each of these two levels are not significantly different at the 5% level. Residual standard deviations ranged from 0.462 (8 df) to 0.590 (10 df).

**Populations.** Five factors had a significant effect on the biofilm's bacterial population (Table 5). On stainless steel, the *L. monocytogenes* populations were always smaller than those on the polymers. The differences between *L. monocytogenes* populations on stainless steel and on polymers were greater for pure cultures than for mixed cultures. As expected, chemical shock slightly reduced the bacterial population. *L. monocytogenes* populations depended on which other species it was associated with: the *L. monocytogenes* population was minimal with strain CCL 63 (log CFU $cm^{-2}$ = 4.7) and maximal with *K. varians* (log CFU $cm^{-2}$ = 5.7). In the latter case, *L. monocytogenes* reached the same mean population as in pure culture. Finally, populations of *L. monocytogenes* in pure culture and of non-*Listeria* strains significantly increased with biofilm age.

## DISCUSSION

As proposed by several authors (7, 22, 50), we have used the term "biofilm" to refer to the community formed by all microorganisms adhering to a substratum, whether single cells or those included in microcolonies.

Most literature reports on bacterial transfer indicate monophasic detachment kinetics (13, 42, 49). We sometimes observed such kinetics ($k_1 = k_2$), but in most cases the curves were biphasic. Among the biphasic curves, those with a negative slope $k_1$ and those with a positive slope $k_1$ can be distinguished. To our knowledge, such positive slopes have never been described before, and they are particularly related to the detachment of *L. monocytogenes* after chemical shock. This seems to indicate that contact with the agar destabilizes the biofilm and results in the detachment of more cells at the next contact. In curves with negative $k_1$, $k_2$ was generally larger than $k_1$, as seen in a previous study in which the two factors studied were the substrate material and the microbial species (35). The

same type of biphasic curve was observed in a field study in which numerous swab samples were taken from the same region of a surface at a catering site (data not shown). This type of curve indicates heterogeneity of biofilm adhesion strength: a first, and usually smaller, subpopulation detaches more easily than the second subpopulation.

To assess adhesion strength, in addition to the slopes $k_1$ and $k_2$, we calculated a TC by dividing the number of cells detached during the first eight contacts by the biofilm population evaluated, using the formula of Veulemans et al. (49). We chose eight contacts so as to have a large number of experimental points. Note, however, that this TC clearly differed from the one we would have obtained by using a single contact. For example, consider the results obtained for one of the units of the fractional design with *L. monocytogenes* in pure culture (for a 1-day biofilm on PVC with the addition of calcium, $k_1 = k_2 = -0.07$). The TC was 0.73 when calculated with the sum of the eight contacts or 0.12 if we considered just the first contact. This coefficient decreased progressively as the number of contacts increased and reached 0.02 at the 12th contact.

Chemical shocks had the clearest effect on adhesion strength. In most cases, the CFU that resisted the shock had high adhesion strengths. Given that chemical shocks also led to population decline, we could have hypothesized a link between population and adhesion strength. Using cells, not cultured as in the present work but deposited on various surfaces 15 min before transfer, Montville and Schaffner (36) showed that the values of transfer coefficients were lower when more CFU were deposited. Considering all 160 populations calculated in our study, there was no link between the biofilm population and the parameters defining adhesion strength ($k_1$, $k_2$, and TC) (data not shown).

By considering all surface bacteria detectable by image analysis (i.e., those cultivable and noncultivable on TSA in 24 h at 30°C), we found in a recent study (34) that the glutaraldehyde-based product (Galox Azur) had the expected effect of fixing the biofilms (aldhehydes have a well-known fixative action, obtained by protein reticulation). We noted this effect in the present study, but it was significant only with the biofilms containing two different species of microorganisms.

In our study of microscopically detectable cells (34), the chlorinated alkaline product differed in its effect from one bacterial strain to another: it slightly increased the adhesion strength of *S. sciuri* but lowered that of *P. fluorescens*. In view of literature data indicating that chlorinated alkaline products (10) or sodium hypochlorite (26) removes biofilm-forming bacteria, at least partially, we concluded that the behavior of *S. sciuri* was rather exceptional. Here, the effect was clear: those CFU that resisted the chlorinated alkaline product had higher adhesion strengths than in the absence of chemical shock, both for *L. monocytogenes* in pure and mixed culture and for the non-*Listeria* strains associated with *L. monocytogenes*. Let us consider the case of *P. fluorescens*. In our previous study (34), we observed that the chlorinated alkaline product (the same product at the same concentration as the one used here) decreased attachment of the *P. fluorescens* cells that were visible under the microscope by 50%. On the other hand, in the current study, mean CFU counts of *P. fluorescens* decreased by 94% when treated by Galorox JH (results not shown). We considered that 1 cell equaled 1 CFU in nontreated biofilms, which is what we found in several other studies, such as that of Leriche and Carpentier) (28) and that we had no more than one layer of cells. Indeed, the biofilms had little nutrient matter available because they were not kept immersed in the culture medium; the nutrients available were provided by the conditioning film obtained by a 75-min immersion in meat exudate, followed by a rinse to remove the nonadhering bacteria. This resulted in a biofilm that could therefore be correctly described by a two-dimensional analysis. A calculation showed that, with an initial *P. fluorescens* population of 7.1 log CFU/cm$^2$ (mean calculated from our results; results not shown), detachment of 50% of cells leaves 6.8 log cells/cm$^2$ on the substratum, whereas a 94% CFU decrease leaves 5.9 log CFU/cm$^2$. We could therefore infer that the difference between 6.8 and 5.9 (that is, 6.7 log cells/cm$^2$) consisted of nonculturable cells. It means that around one of seven visible cells were nonculturable cells; the results obtained in our previous study concerned mainly nonculturable cells. We can therefore hypothesize for *P. fluorescens* that only the cultivable cells (the number of CFU on TSA) had increased adhesion strength after treatment with chlorinated alkaline product. However, this cleaner-disinfectant has a recognized capacity to remove bacteria, so we may also hypothesize that the culturable cells that were not detached represented a third minority subpopulation of culturable cells that were "superresistant" (6, 30) and "super adhesive."

The second significant factor we observed was the nature of the non-*Listeria* strain associated with *L. monocytogenes*. Previous studies (8, 27, 33) showed that overall, there were lower levels of *L. monocytogenes* colonization of the surface in the presence of three of the four non-*Listeria* strains studied: CCL 63, *S. sciuri*, and *P. fluorescens*. Strain CCL 63 had the most pronounced effect; overall, *K. varians* had a favorable effect on the *L. monocytogenes* population. In the present study, we also found such a trend, and *K. varians* was associated with the largest mean population of *L. monocytogenes*, whereas strain CCL 63 was associated with the lowest. There was no difference between populations of *L. monocytogenes* in pure culture or cultured in the presence of *K. varians.* Given that the two species must share available nutrients and so must have lower populations by the end of their growth than when cultured alone, *K. varians* is likely to have a favorable effect on the colonization of *L. monocytogenes* on the substrates. We have observed that *L. monocytogenes* cultivated on stainless steel in the presence of *K. varians* grew very distinctly around *K. varians* microcolonies (8). It was also in the presence of *K. varians* that we observed the highest transfer coefficient of *L. monocytogenes* (0.78), compared with the average transfer coefficient of 0.55 for *L. monocytogenes* alone. This shows that *K. varians* favors both the attachment and detachment of *L. monocytogenes*.

The substrate material had a clear effect on the biofilm population which, as noted previously (35), was always smaller on stainless steel. Only the non-*Listeria* strains associated with *L. monocytogenes* showed low adhesion strengths on stainless steel. With our previous study (35), we also observed greater $k_2$ values (low adhesion strengths) with stainless steel, and the effect was also visible with *L. monocytogenes* in pure culture, which was not the case with the present study.

Biofilm age was investigated because of the increased strength of attachment of a *P. fluorescens* biofilm observed

between days 1 and 4 (unpublished data). One-day biofilms were chosen to mimic what could happen in a factory where cleaning and disinfection operations are performed once a day. Two-day biofilms were chosen to mimic a site that had been forgotten once during the cleaning and disinfection of such a factory. Increase in biofilm age led to a rise in the mean population of the non-*Listeria* strains associated with *L. monocytogenes* and of *L. monocytogenes* in pure culture. The increase was likely due to an accumulation of cells but also to the fact that resistance to antimicrobials increases with the age of the biofilm (16). Biofilm age did not have any effect on adhesion strength, probably because the two levels of this factor (1 and 2 day) were too close to each other.

The addition of calcium and glucose, factors that are often studied together (2, 31, 51), was chosen because they can enhance the cohesion of the biofilm by promoting the formation of calcium bridges between the negatively charged polymers (14) and the synthesis of exopolysaccharides by the biofilm, respectively. Addition of calcium did not have any effect on mixed biofilm populations, although added calcium was shown to increase adhesion of pure culture biofilms made using one of the four non-*Listeria* strains used here (34). Furthermore, addition of calcium to mixed biofilms had no visible effect on attachment strength, probably because the effect was dependent on the strain. Indeed, increased formation of microcolonies that are preferentially detached on contact was seen only with *S. sciuri* and *P. fluorescens* (34).

The practical consequences of the present findings can be illustrated by taking the case of slices of ham which are successively in contact with the same surface of a conveyor belt contaminated by *L. monocytogenes*. Two types of information are needed to assess the dose of *L. monocytogenes* on the slices of ham and hence the risk of listeriosis: (i) the number of CFU that are transferred to each ham slice and (ii) the number of slices that will be contaminated. The infectious nature of *L. monocytogenes* is linked to its dose; if the degree of contamination is low and initially unlikely to provoke an infection, storage at high temperature and/or for a long period may enable the pathogenic bacterium to proliferate to significant population levels. The number of cells transferred, at least with the first contact, depends on the biofilm population. This is clear from the two following extreme examples. If we consider an experimental unit (a 1-day mixed biofilm where *L. monocytogenes* was cultivated with *K. varians* on polyurethane [PU2] with the addition of calcium and glucose) where a large *L. monocytogenes* population of 7.76 log CFU $cm^{-2}$ was associated with a low transfer coefficient (0.026), $1.5 \times 10^6$ *L. monocytogenes* CFU $cm^{-2}$ were detached by eight contacts. On the other hand, if we consider a second experimental unit (a 2-day biofilm of *L. monocytogenes* alone grown on stainless steel with the addition of glucose and treated with a glutaraldehyde-based product) where a small population of 2.89 log CFU $cm^{-2}$ was associated with a high transfer coefficient (0.85), $6.6 \times 10^2$ CFU $cm^{-2}$ were detached by eight contacts.

On the other hand, we have seen that the number of contacts needed to leave just 1 CFU $cm^{-2}$ depends mainly on the slope $k_2$. The number of slices of ham contaminated, therefore, also depends mainly on the slope $k_2$. So, to lower the immediate risk, it is necessary to reduce the biofilm population; to decrease the delayed risk, it is also necessary to diminish the slope $k_2$ (decrease the adhesion strength). Cleaning and disinfection agents (such as chlorinated-alkaline products that are mainly used as cleaning agents in the presence of proteinaceous soiling) reduce biofilm populations by placing part of the microbial cells in suspension. Disinfection agents aim at killing microbial cells that were not removed by the previous cleaning. In general, these products, used sequentially, are effective at reducing both the immediate and delayed risks because no more living *L. monocytogenes* cells are left on the surface. Of course, neither the low sanitizer concentrations nor the *L. monocytogenes* populations used here are representative of real situations. Such conditions were chosen to detect enough residual CFU to build a transfer curve. However, cleaning and disinfection operations may be effective only against the immediate risk. Yet incidents where *L. monocytogenes* cells remain on surfaces, despite multiple cleaning and disinfection procedures, have been frequently reported (15, 47); such *L. monocytogenes* strains are called persistent. It is of some importance to remember that the aging of biofilms, since it causes an increased resistance to disinfectants (10), is likely to be a factor in contributing to persistence. It would, therefore, be interesting to find ways to decrease delayed risk. The effect of a glutaraldehyde-based disinfectant on the slope $k_2$ is clear: it increases adhesion strength and hence the subsequent risk. We may hypothesize that the same effect occurs at high concentration, because of the well-known fixing power of aldehydes. Concerning the chlorinated alkaline product used at low concentrations, our data do not show whether it has a direct effect on adhesion strength of culturable cells or whether it simply leads to the emergence of a superadhesive culturable subpopulation. We can also not conclude what would have happened if the recommended concentration had been used. What is clear is that as shown in our previous study, with pure *L. monocytogenes* biofilms (35), only the use of stainless steel clearly appears to reduce both the culturable population and the slope $k_2$, and hence the adhesion strength of culturable cells. The present study gives at least one of the possible reasons why it is impossible to eliminate surface microorganisms (persistent *L. monocytogenes* but, above all, *Pseudomonadaceae*) completely by means of the cleaning and disinfection procedures conventionally used in the food industry (32).

Finally, the behavior of *L. monocytogenes* in mixed biofilms differs from that in pure culture: the effect of the substrate material on the population is attenuated in mixed culture; most of all, the adhesion strength of *L. monocytogenes* depends on the non-*Listeria* strain with which it is combined. However, the current study was conducted with only one strain of *L. monocytogenes*. It would be interesting to test other *L. monocytogenes* strains and particularly strains belonging to a different lineage, as biofilm formation appears to be different according to the lineage of the strain used (4, 12).

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